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| (51) International Patent Classification ⁵ : A01N 43/42, A61K 31/47 | A1 | (11) International Publication Number: WO 94/28721 (43) International Publication Date: 22 December 1994 (22.12.94) |
| (21) International Application Number: PCT/US94/06203 (22) International Filing Date: 2 June 1994 (02.06.94) (30) Priority Data: 08/076,312 11 June 1993 (11.06.93) US 08/184,519 21 January 1994 (21.01.94) US (71) Applicant: THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY [US/US]; Suite 350, 900 Welch Road, Stanford, CA 94303 (US). (72) Inventors: COOKE, John, P.; 4022 Ben Lomond Drive, Palo Alto, CA 94306 (US). DZAU, Victor, J.; 12101 Dawn Lane, Los Altos Hills, CA 94022 (US). GIBBONS, Gary, H.; 3212 Morris Drive, Palo Alto, CA 94303 (US). (74) Agents: ROWLAND, Bertram, I. et al.; Flehr, Hohbach, Test, Albritton & Herbert, Suite 3400, 4 Embarcadero Center, San Francisco, CA 94111-4187 (US). | (81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> | |
| (54) Title: TREATMENT OF VASCULAR DEGENERATIVE DISEASES BY MODULATION OF ENDOGENOUS NITRIC OXIDE PRODUCTION OR ACTIVITY (57) Abstract Atherogenesis and restenosis are treated by long term administration of physiologically acceptable compounds which enhance the level of endogenous nitric oxide or other intermediates in the NO induced relaxation pathway in the host. Alternatively, or in combination, other compounds may be administered which provide for short term enhancement of nitric oxide, either directly or by physiological processes. In addition, cells may be genetically engineered to provide a component in the synthetic pathway to nitric oxide, so as drive the process to enhance nitric oxide concentration, particularly in conjunction with the administration of a nitric oxide precursor. | | |

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**TREATMENT OF VASCULAR DEGENERATIVE DISEASES BY
MODULATION OF ENDOGENOUS NITRIC OXIDE PRODUCTION OR
ACTIVITY**

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INTRODUCTION

This invention was supported in part by the United States Government under Grant 1K07HCO2660 (NHLBI). The U.S. Government may have an interest in this application.

15 **Technical Field**

The field of this invention is the modulation of NO activity, which finds application in the treatment of vascular degenerative diseases, particularly atherosclerosis and restenosis.

20 **Background**

Atherosclerosis and vascular thrombosis are a major cause of morbidity and mortality, leading to coronary artery disease, myocardial infarction, and stroke. Atherosclerosis begins with an alteration in the endothelium, which lines the blood vessels. The endothelial alteration
25 results in adherence of monocytes, which penetrate the endothelial lining and take up residence in the subintimal space between the endothelium and the vascular smooth muscle of the blood vessels. The monocytes

absorb increasing amounts of cholesterol (largely in the form of oxidized or modified low-density lipoprotein) to form foam cells. Oxidized low-density lipoprotein (LDL) cholesterol alters the endothelium, and the underlying foam cells distort and eventually may even rupture through the
5 endothelium.

Platelets adhere to the area of endothelial disruption and release a number of growth factors, including platelet derived growth factor (PDGF). PDGF, which is also released by foam cells and altered endothelial cells, stimulates migration and proliferation of vascular smooth muscle cells into
10 the lesion. These smooth muscle cells release extracellular matrix (collagen and elastin) and the lesion continues to expand. Macrophages in the lesion elaborate proteases, and the resulting cell damage creates a necrotic core filled with cellular debris and lipid. The lesion is then referred to as a "complex lesion." Rupture of this lesion can lead to
15 thrombosis and occlusion of the blood vessel. In the case of a coronary artery, rupture of a complex lesion may precipitate a myocardial infarction, whereas in the case of a carotid artery, stroke may ensue.

One of the treatments that cardiologists and other interventionalists employ to reopen a blood vessel which is narrowed by plaque is balloon
20 angioplasty (approximately 300,000 coronary and 100,000 peripheral angioplasties are performed annually). Although balloon angioplasty is successful in a high percentage of the cases in opening the vessel, it unfortunately denudes the endothelium and injures the vessel in the process. This damage causes the migration and proliferation of vascular
25 smooth muscle cells of the blood vessel into the area of injury to form a lesion, known as myointimal hyperplasia or restenosis. This new lesion leads to a recurrence of symptoms within three to six months after the angioplasty in a significant proportion of patients (30-40%).

Because of their great prevalence and serious consequences, it is
30 critically important to find therapies which can diminish the incidence of atherosclerosis, vascular thrombosis and restenosis. Ideally, such therapies would inhibit the pathological processes associated with

atherosclerosis, thereby providing prophylaxis or retarding the progression of the degenerative process.

As briefly summarized above, these pathological processes are extremely complex, involving a variety of different cells which undergo changes in their character, composition, and activity, as well as in the nature of the factors which they secrete and the receptors that are up- or down-regulated. A substance released by the endothelium, "endothelium derived relaxing factor" (EDRF), may play an important role in inhibiting these pathologic processes. EDRF is now known to be nitric oxide (NO) or a labile nitroso compound which liberates NO. (For purposes of the subject invention, unless otherwise indicated, nitric oxide (NO) shall intend nitric oxide or the labile precursor.) This substance has been reported to relax vascular smooth muscle, inhibit platelet aggregation, inhibit mitogenesis and proliferation of cultured vascular smooth muscle, and leukocyte adherence. NO may have other effects, either direct or indirect, on the various cells associated with vascular walls and degenerative diseases of the vessel.

Relevant Literature

Girerd, et al. (1990) Circulation Research 67, 1301-1308 report that intravenous administration of L-arginine potentiates endothelium-dependent relaxation in the hind limb of cholesterol-fed rabbits. The authors conclude that synthesis of EDRF can be increased by L-arginine in hypercholesterolemia. Rossitch, et al. (1991) J. Clin. Invest. 87, 1295-1299 report that *in vitro* administration of L-arginine to basilar arteries of hypercholesterolemic rabbits reverses the impairment of endothelium-dependent vasodilation and reduces vasoconstriction. They conclude that the abnormal vascular responses in hypercholesterolemic animals is due to a reversible reduction in intracellular arginine availability for metabolism to nitric oxide.

Creager, et al. (1992) J. Clin. Invest. 90, 1248-1253, report that intravenous administration of L-arginine improves endothelium-derived NO-dependent vasodilation in hypercholesterolemic patients.

Cooke, et al., "Endothelial Dysfunction in Hypercholesterolemia Is Corrected by L-arginine," Endothelial Mechanisms of Vasomotor Control, eds. Drexler, Zeiher, Bassenge, and Just; Steinkopff Verlag Darmstadt, 1991, pp. 173-181, review the results of the earlier references and
5 suggest, "If the result of these investigations may be extrapolated, exogenous administration of L-arginine (i.e., in the form of dietary supplements) might represent a therapeutic adjunct in the treatment and/or prevention of atherosclerosis."

Cooke, (1990) Current Opinion in Cardiology 5, 637-644 discusses
10 the role of the endothelium in the atherosclerosis and restenosis, and the effect that these disorders have on endothelial function.

Cooke (1992) J. Clin. Invest. 90, 1168-1172, describe the effect of chronic administration of oral L-arginine in hypercholesterolemic animals on atherosclerosis. This is the first demonstration that oral L-arginine
15 supplements can improve the release of NO from the vessel wall. The increase in NO release from the vessel wall was associated with a striking reduction in atherosclerosis in hypercholesterolemic animals. This is the first evidence to support the hypothesis that increasing NO production by the vessel wall inhibits the development of atherosclerosis.

Cooke and Tsao, (1992) Current Opinion in Cardiology 7, 799-804
20 describe the mechanism of the progression of atherosclerosis and suggest that inhibition of nitric oxide may disturb vascular homeostasis and contribute to atherogenesis.

Cooke and Santosa, (1993) In: Steroid Hormones and Dysfunctional
25 Bleeding, AAAS Press, review the activities of EDRF in a variety of roles and suggest that reversibility of endothelial dysfunction may be affected by the stage of atherosclerosis. They conclude that EDRF is a potent vasodilator, plays a key role in modulating conduit and resistance vessel tone, has important effects on cell growth and interactions of circulatory
30 blood cells with a vessel wall, and that disturbances of EDRF activity may initiate or contribute to septic shock, hypertension, vasospasm, toxemia and atherosclerosis.

Fitzpatrick, et al., American Journal of Physiology 265 (Heart Circ. Physiol. 34):H774-H778, 1993 report that wine and other grape products may have endothelium-dependent vasorelaxing activity *in vitro*.

Other references which refer to activities attributed to NO or its precursor include: Pohl and Busse (1989) Circ. Res. 65:1798-1803; Radomski et al. (1987) Br. J. Pharmacol. 92:181-187; and Stamler et al. (1989) Circ. Res. 65:789-795; anti-platelet activity); Garg and Hassid (1989) J. Clin. Invest. 83:1774-1777; and Weidinger et al, (1990) Circulation 81:1667-1679; (NO activity in relation to vascular smooth muscle growth); Ross (1986) N. Engl. J. Med. 314:488-500; Bath et al. (1991) Arterioscler. Thromb. 11:254-260; Kubes et al. (1991) Proc. Natl. Acad. Sci. USA 89:6348-6352; Lefer et al. (1990) In: Endothelium-Derived Contracting Factors. Basel, S. Karger, pp.190-197; (NO activity in relation to leukocyte adhesion and migration); Heistad et al. (1984) Circ. Res. 43:711-718; Rossitch et al. (1991) J. Clin. Invest. 87:1295-1299; Yamamoto et al. (1988) *ibid* 81:1752-1758; Andrews et al. (1987) Nature 327:237-239; Tomita et al. (1990) Circ. Res. 66:18-27; Kugiyama et al. (1990) Nature 344:160-162; Mitchell et al. (1992) J. Vasc. Res. 29:169 (abst.); and Minor et al. (1990) J. Clin. Invest. 86:2109-2116; (NO activity in relation to hypercholesterolemia); Tanner et al. (1991) Circulation 83:2012-2020; Kuo et al. (1992) Circ. Res. 70:f465-476; Drexler et al. (1991) Lancet 338:1546-1550; and Nakanishi et al. (1992) In: Scientific Conference on Functional and Structural Mechanisms of Vascular Control, Snowbird, UT, p. 86 (abstr.); relation of L-arginine to NO-dependent vasodilation.

SUMMARY OF THE INVENTION

Methods are provided for modulating cellular adhesion, infiltration and proliferation by modulating the level of nitric oxide or active precursor at a physiological site. The methods find use in the treatment of atherosclerosis and restenosis. The enhancement of endogenous nitric oxide or secondary messenger availability at a physiological site inhibits the progression of restenosis and atherosclerosis. As a prophylaxis or

treatment for atherosclerotic susceptible hosts, the agent is chronically administered at an effective dosage. For restenosis, the agent may be administered for a limited period since this pathological process generally abates 3-6 months after the vascular injury (i.e. angioplasty or
5 atherectomy). Oral administration of L-arginine as a dietary supplement will increase NO elaboration and thereby diminish the effects of atherogenesis. Other techniques to enhance NO or secondary messenger availability may be utilized such as increasing the availability of NO synthase. For example, enhanced expression of NO synthase in the
10 vessel wall, particularly at the lesion site, results in the release of NO from the vessel wall or a reduction in the degradation of NO or the secondary messenger, cyclic guanosine monophosphate ("cGMP").

BRIEF DESCRIPTION OF THE DRAWINGS

15 Fig. 1 is a bar diagram of histomorphometric studies of the effect of L-arginine on atherosclerotic plaque in hypercholesterolemic animals (See Ex. 1);

Fig. 2 is a nephelometric scan of the effect of L-arginine diet supplement on platelet reactivity as evidenced by platelet aggregation
20 initiated by adenosine diphosphate (See Ex. 2);

Fig. 3 is a bar diagram comparing the effect of L-arginine diet supplement on cell binding to aortic endothelium of hypercholesterolemic animals (See Ex. 4); and

Fig. 4 is a bar diagram of morphometric measurements of intimal
25 lesion thickness in EC-NOS treated animals in comparison to control vector or untreated injured vessels (See Ex. 6)

DESCRIPTION OF SPECIFIC EMBODIMENTS

In accordance with the subject invention, common vascular
30 degenerative diseases such as atherosclerosis, vascular thrombosis, and restenosis, are treated prophylactically and/or therapeutically by enhancing the physiological signal resulting from cellular response to endothelium-derived relaxing factor ("EDRF"), e.g. by maintaining an enhanced level of

nitric oxide, its precursor, or a secondary messenger associated with the relaxation signal, e.g. cyclic guanosine monophosphate ("cGMP"), at the vessel wall in accordance with a predetermined regimen over an extended period of time. The enhanced level of nitric oxide (which is intended to include any precursor of nitric oxide which results in such enhanced level) can be achieved by modulating the activity, synthesis or concentration of any of the components associated with the formation of nitric oxide in the nitric oxide synthetic pathway, or inhibiting the rate of degradation of nitric oxide, its precursors, or the secondary messengers associated with the relaxation signal. The enhanced level of nitric oxide may be a result of administration to the patient of an intermediate in the metabolic pathway to the production of nitric oxide (or its physiological equivalent), the enhanced levels of an enzyme associated with the production of nitric oxide, or a physiologically acceptable precursor, which may lead directly or indirectly, to formation of nitric oxide. The agent will be administered in a form of other than a natural food source, such as meat or plants as natural protein sources, fruits or products derived therefrom, e.g. wine, or the like.

One approach is to employ L-arginine as a dietary supplement. This amino acid may be administered as any physiologically acceptable salt, such as the hydrochloride salt, glutamate salt, etc. It may also be administered as a peptide (i.e. poly-L-arginine) so as to increase plasma levels of the NO precursor. Naturally occurring sources include protamine. The administration of L-arginine or other convenient NO precursor would be in accordance with a predetermined regimen, which would be at least once weekly and over an extended period of time, generally at least one month, more usually at least three months, as a chronic treatment, and could last for one year or more, including the life of the host. The dosage administered will depend upon the frequency of the administration, the blood level desired, other concurrent therapeutic treatments, the severity of the condition, whether the treatment is for prophylaxis or therapy, the age of the patient, the natural level of NO in the patient, and the like. Desirably, the amount of L-arginine or biologically equivalent compound

which is used would generally provide a plasma level in the range of about 0.2mM to 30mM. The oral administration of L-arginine can be achieved by providing L-arginine as a pill, powder, capsule, liquid solution or dispersion, particularly aqueous, or the like. Various carriers and
5 excipients may find use in formulating the NO precursor, such as lactose, terra alba, sucrose, gelatin, aqueous media, physiologically acceptable oils, e.g. peanut oil, and the like. Usually, if daily, the administration of L-arginine for a human host will be about 1 to 12g per day.

The administration of L-arginine may be administered
10 prophylactically, so as to inhibit atherogenesis or restenosis, or therapeutically after atherogenesis has been initiated. Thus, for example, a patient who is to undergo balloon angioplasty may have a regimen of L-arginine administered substantially prior to the balloon angioplasty, preferably at least about a week or substantially longer. Alternatively, in a
15 patient where atherogenesis is suspected, the administration of L-arginine may begin at any time. Of particular interest is the incorporation of L-arginine as a supplement in a food, such as a health bar, e.g. granola, other grains, fruit bars, such as a date bar, fig bar, apricot bar, or the like. The amount of L-arginine or the equivalent would be about 2 - 25 g per
20 dosage or bar, preferably about 3 - 15 g.

Instead of oral administration, intravascular administration may also be employed, particularly where more rapid enhancement of the nitric oxide level in the vascular system is desired (i.e. as with acute thrombosis of a critical vessel), so that combinations of oral and parenteral
25 administrations may be employed in accordance with the needs of the patient. Furthermore, parenteral administration may allow for the administration of compounds which would not readily be transported across the mucosa from the gastrointestinal tract into the vascular system.

30 Another approach is to administer the active ingredient of grape skin extract. See Fitzpatrick et al. (1993), supra. The extract may be enriched for the active component by employing separation techniques and assaying the activity of each of the fractions obtained. The grape

skin extract may be divided into fractions using in a first gel permeation separation to divide the extract by the size of the components. The active fraction(s) may be determined by an appropriate assay, see the experimental section. The active fraction(s) may be further separated using HPLC and an appropriate eluent, conveniently either an isocratic eluent of aqueous acetonitrile or propanol or a linearly varying eluent, using the same solvents. Fractions which are shown to be active and substantially pure, e.g. at least 80 weight %, by thin layer chromatography, mass spectrometry, gas phase chromatography, or the like may then be characterized using infra-red, nuclear magnetic resonance, mass or other spectroscopy.

For intravascular administration, a wide variety of individual or combinations of physiologically acceptable compositions may be employed, which may be provided systemic administration or in proximity to a lesion site. Thus, one may provide for combinations of peroxy compounds or other oxygen containing oxidants, where the oxidant is physiologically acceptable, in conjunction with a nitrogen source, such as substituted guanidines, e.g. L-arginine, amidines, or the like. Alternatively, one may reduce the degradation of endogenous nitric oxide using antioxidants or scavengers of oxygen-derived free radicals (such as sulfhydryl containing compounds) or compounds that prevent the production of oxygen-derived free radicals (such as superoxide dismutase), as it is known that oxygen-derived free radicals (such as superoxide anion) can inactivate nitric oxide. Thiol compounds may also find application, as well as their derivatives, such as disulfides, sulfonic acids, thiol esters, thiono thiol esters, and the like. Other compounds which may find use include partially oxidized nitrogen compounds, such as hydroxylamines, oxazoles, oxazines, nitroso compounds, or the like. Physiologically acceptable stable free radical compounds, such as nitroxyl compounds, may find use, where the unpaired electron may be on nitrogen or oxygen, analogous to NO. These compounds will be, for the most part, synthetic organic compounds generally having a molecular weight of at least about 100 and usually not more than about 2000D.

Other compositions which may find use include nitrites, including nitrite esters, e.g. esters of carbonate, thiocarbonate, etc. These compositions may be administered at the site of a lesion to provide for rapid enhancement of nitric oxide concentration, so as to initiate or inhibit the various physiological processes affected by the level of nitric oxide present and associated with plaque formation or restenosis. Particularly, processes associated with the vascular smooth muscle ("VSM") cell proliferation and invasion of the endothelial layer can be modulated.

Alternatively, one can enhance, either in conjunction with the enhancement of precursors to nitric oxides or independently, components of the nitric oxide metabolic pathway. For example, one may enhance the amount of nitric oxide synthetase present in the vessel wall, particularly at the site of lesions. This can be done by local administration to the lesion site or systemically into the vascular system. The synthase may be administered using liposomes, slow release particles, or in the form of a depot, e.g. in collagen, hyaluronic acid, biocompatible gels, vascular stents, or other means, which will provide the desired concentration of the NO-synthase at the lesion site.

Instead of providing for the enhancement of NO at the physiological site of interest, one may choose to extend the lifetime of the signal transduced as a result of the presence of nitric oxide. Since cGMP is produced intracellularly as a result of a nitric oxide induced signal, employing agents which result in the production of or extending the lifetime of cGMP may be employed. Illustrative agents include cGMP phosphodiesterase inhibitors or agents which increase the amount of guanylate cyclase. Alternatively, one may intervene at the level of protein kinase C phosphorylation to provide the desired relaxation signal.

Alternatively, cells may be genetically engineered to provide for constitutive or inducible expression of one or more genes, which will provide for the desired relaxation response, by expressing NO synthase, or other enzymes or proteins which can be secreted and act extracellularly. Thus, expression vectors (viral or plasmid) may be prepared which contain the appropriate gene(s) and which can be introduced into host cells which

will then produce high concentrations of nitric oxide or other intermediate in the relaxation pathway. These cells may be introduced at the lesion site or at another site in the host, where the expression will induce the appropriate response as to relaxation, proliferation, etc. The NO synthase or cells expressing the NO synthase may be present as depots by encapsulation and positioning at the site of interest. For example, porous stents may be produced which encapsulate the enzyme or cells to protect the enzyme from degradation and removal or, for the cells, to protect the cells from the immune system.

10 Cultured cells can be transfected with expression vectors containing the NO synthase or other gene ex-vivo and then introduced into the vessel wall, using various intra-arterial or intra-venous catheter delivery systems. Alternatively, techniques of in vivo gene transfer can be employed to transfect vascular cells within the intact vessel in vivo. The gene(s) can be expressed at high constitutive levels or can be linked to an inducible promoter (which may have tissue specificity) to allow for regulation of expression.

DNA constructs are prepared, where the appropriate gene, e.g. a NO synthase gene, is joined to an appropriate promoter, either with its native termination region or a different termination region, which may provide for enhanced stability of the messenger RNA. Constitutive promoters of particular interest will come from viruses, such as Simian virus, papilloma virus, adenovirus, HIV, Rous sarcoma virus, cytomegalovirus or the like, where the promoters include promoters for early or late genes, or long terminal repeats. Endogenous promoters may include the β -actin promoter, or cell-type specific promoters.

A construct is prepared in accordance with conventional techniques, the various DNA fragments being introduced into an appropriate plasmid or viral vector, normally a vector capable of replication in a bacterial and/or eucaryotic host. Normally, the vector will include a marker, which allows for selection of cells carrying the vector, e.g. antibiotic resistance. The vector will normally also include an origin which

is functional in the host for replication. Other functional elements may also be present in the vector.

Once the vector has been prepared and replicated, it may then be used for introduction into host cells. The plasmid vector construct may be
5 further modified by being joined to viral elements which allow for ease of transfection, may provide a marker for selection, e.g. antibiotic resistance, or other functional elements. Introduction of the plasmid vector construct into the host cells may be achieved by calcium phosphate precipitated
10 DNA, transfection, electroporation, fusion, lipofection, viral capsid-mediated transfer, or the like. Alternatively, the expression construct within viral vectors may be introduced by standard infection techniques. For somatic cell gene therapy, autologous cells will generally be employed, although in some instances allogeneic cells or recombinantly modified cells may be employed. Usually the cells employed for genetic modification will
15 be mature endothelial or vascular smooth muscle cells. Occasionally, the cells employed for genetic modification will be progenitor cells, particularly early progenitor cells. For example, myoblasts may be employed for muscle cells or hematopoietic stem cells or high proliferative potential cells may be employed for lymphoid and/or myelomonocytic cells.

20 Depending upon the nature of the cells, they may be injected into tissue of the same or different cellular nature, they may be injected into the vascular system, where they may remain as mobile cells or home to a particular site (i.e. the lesion). For the NO synthase gene, the number of cells which are administered will depend upon the nature of the cells, the
25 level of production of the NO synthase, the desired level of NO synthase in the host vascular system, at the lesion site, or the like, whether the enhanced level of NO synthase is the only treatment or is used in conjunction with other components of the nitric oxide synthetic pathway, and the like. Therefore, the particular number of cells to be employed will
30 be determined empirically in accordance with the requirements of the particular patient.

These cells may also be introduced into the circulation by first growing them on the surface of standard vascular graft material (i.e.

Dacron or polytetrafluoroethylene grafts) or other synthetic vascular conduits or vascular bioprostheses. In this way, the cells may be introduced into the host by introducing the vascular graft seeded with the cells.

5 Alternatively, one may use viral vectors, which are capable of infecting cells *in vivo*, such as adenovirus or retroviruses. In this case, the viral vector containing the NO synthase gene or other gene involved with the relaxation pathway will be administered directly to the site of interest, where it will enter into a number of cells and become integrated
10 into the cell genome. Thus, one can titer the desired level of nitric oxide synthase which is secreted or other protein which is expressed, by providing for one or more administrations of the virus, thus incrementally increasing the amount of synthase which is secreted or other protein which is produced.

15 Alternatively, one may use modified liposomes as a vehicle for endovascular administration of the vector containing the NO synthase or other gene. One such modified liposome technique involves mixing the liposomes with the vector containing NO synthase. Once the gene expression construct-containing vector is incorporated into the liposome,
20 the liposomes are coated with a protein (e.g. the viral coat protein of the Hemagglutinating Virus of Japan) that increases the affinity of the liposome for the vessel wall.

 In some situations, the NO synthase or other gene in the relaxation pathway may be co-transfected with an artificial gene encoding an
25 arginine rich polypeptide susceptible to proteolytic cleavage as an intracellular source of L-arginine. In other situations, the NO synthase or other gene may be co-transfected with the superoxide dismutase gene, so as to inhibit the degradation of the nitric oxide.

 In some situations, acute treatment may be involved, requiring one
30 or a few administrations. This will normally be associated with compounds which can act as nitric oxide precursors and are other than naturally occurring compounds or are compounds which may be added with naturally occurring compounds to enhance the rate of formation of

nitric oxide. Thus, one may provide for acute administration of L-arginine and superoxide dismutase to increase the nitric oxide concentration over a restricted period of time. These administrations may be independent of or in conjunction with long, term regimens.

- 5 The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Example 1. Anti-atherogenic effects of oral arginine:

- 10 **Study design:** (See, Cooke et al., 1992, *supra*) Male New Zealand white rabbits (n=49) were assigned to one of three treatment groups: 10 were fed with normal rabbit chow for ten weeks (Control); 19 received chow enriched with 1% cholesterol (Chol); and 20 received a 1% cholesterol diet supplemented with 2.5% L-arginine hydrochloride in the
15 drinking water (Arg.). Following ten weeks of the dietary intervention, animals were lightly sedated and the central ear artery cannulated for measurement of intra-arterial blood pressure, followed by collection of blood samples for serum chemistries and plasma arginine. Subsequently the animals were sacrificed and the left main coronary artery and the
20 thoracic aorta were harvested for studies of vascular reactivity and histomorphometry. In some animals, blood was collected for studies of platelet and monocyte reactivity.

Results: Biochemical and physiological measurements.

- Hypercholesterolemic animals maintained on oral L-arginine
25 supplementation (Arg) experienced a twofold elevation in plasma arginine levels in comparison to animals on a normal (Control) or 1% cholesterol (Chol) diet alone; the elevation in plasma arginine was maintained throughout the course of the study. Serum cholesterol measurements were elevated equally in both groups receiving the 1% cholesterol diet
30 [50 ± 6 vs. 1629 ± 422 vs. 1852 ± 356 mg/dl respectively for Control (= 10), Chol (= 13), and Arg (= 14)]. There were no significant differences in hemodynamic measurements between groups.

Organ chamber studies of isolated vessels: For NO-independent responses, there were no differences between the treatment groups in maximal response or sensitivity to norepinephrine (a vasoconstrictor), or to nitroglycerin (a nitrovasodilator). By contrast, NO-dependent
5 relaxations were attenuated in vessels harvested from hypercholesterolemic animals with a reduction in the maximal response to acetylcholine, and a reduction in sensitivity to calcium ionophore. In comparison, vessels harvested from hypercholesterolemic animals receiving L-arginine supplementation had improved NO-dependent
10 relaxation. Similarly, sensitivity of vessels to calcium ionophore A23187 was greater in the Arg group. In a separate study, the effect of chronic arginine supplementation to improve NO-dependent relaxation was confirmed in the hypercholesterolemic rabbit abdominal aorta.

15 **Histomorphometric studies (planimetry of EVG-stained sections):** A blinded histomorphometric analysis revealed that medial cross-sectional areas of thoracic aortae were not different between the groups. By contrast, the intimal cross-sectional area (i.e. amount of atherosclerotic plaque) of vessels from hypercholesterolemic animals receiving L-arginine
20 supplementation was reduced in comparison to those from animals receiving cholesterol diet alone. In the Arg animals the reduction in the intimal lesion was most pronounced in the ascending thoracic aorta and left main coronary artery. In the left main coronary artery of hypercholesterolemic animals receiving arginine, essentially no
25 atherosclerotic plaque developed.

Changes in lesion surface area: In a second series of studies, the extent of the thoracic aorta involved by lesions was examined. In hypercholesterolemic rabbits receiving vehicle (n = 6) or L-arginine
30 supplement (n = 6), thoracic aortae (from left subclavian artery to diaphragm) were harvested after ten weeks of treatment, bisected longitudinally, and stained with oil-red O. Vessels were photographed and vessel and lesion surface area determined by planimetry. Approximately

40% of the total surface area was covered with plaque in thoracic aortae from hypercholesterolemic animals receiving vehicle, whereas in thoracic aortae from arginine-treated hypercholesterolemic animals, less than 10% of the surface area was covered with plaque.

- 5 To summarize, dietary arginine supplementation increases plasma arginine levels, but does not alter serum cholesterol. This is associated with significant improvement in NO-dependent vasodilation as judged by bioassay. Finally, the improvement in NO-dependent vasodilation is associated with reduction in thickness and area of the lesions in vessels
10 from hypercholesterolemic animals.

Example 2. Inhibition of platelet aggregation by oral L-arginine:

- The effect of L-arginine supplementation on platelet reactivity in rabbits that had normal chow (Control; n = 6), a 1% cholesterol diet (Chol; n = 5), or a 1% cholesterol diet supplemented with oral arginine (Arg; n = 6), as detailed above, was examined. Arterial blood obtained after central ear artery cannulation was anticoagulated with 13mM sodium citrate. Platelet-rich suspension was prepared by washing platelets in calcium-free Krebs-Henseleit solution and resuspending them in Tyrode's solution with albumin. The platelet count was adjusted at 2.5×10^4 platelets/ μ l by addition of platelet-poor plasma. Aggregation was initiated by addition of adenosine diphosphate and monitored by standard nephelometric techniques. In platelets derived from Chol animals, aggregation was not different in rate or maximum extent in comparison to platelets from Control animals (A). By contrast, aggregation of platelets from Arg animals was reduced by 50% (B).

- This reduction in platelet aggregation was associated with a two-fold greater cGMP content in aggregated platelets from arginine-treated animals. The reduction of platelet reactivity could be reversed by administration of N-methylarginine (10^{-4} M) in vitro (C) (Fig. 2.)
30 Therefore, the anti-platelet effect of chronic oral arginine administration can be credited to an increased synthesis of endogenous NO.

Furthermore, NO synthesis may be induced in both the platelets and the endothelium.

Example 3. Inhibition of monocyte adherence:

- 5 **A. Functional Binding Assay:** To determine if oral arginine supplementation affects monocyte adherence, blood was collected from rabbits fed normal chow (= 6), a 1% cholesterol diet (= 6), or a 1% cholesterol diet supplemented with L-arginine (= 6), as described above. Mononuclear cells were purified from blood by Ficoll-paque density
10 gradient centrifugation. In these preliminary studies, adhesion was examined of blood leukocytes to a transformed endothelial cell line, bEnd3 (mouse brain-derived polyoma middle T antigen transformed endothelial cells). The bEnd3 cells display the morphology of endothelial cells, and like human endothelial cells are capable of uptake of acetylated low-
15 density lipoprotein and express adhesion molecules in a cytokine-regulatable fashion. Cultured cells were grown to confluence in 0.5 cm² Lab-Tek chamber slides (MilesScientific) and treated with control medium or with LPS (1 mg/ml) or TNF α (25 U/ml) for 18 hours. Cultures were washed with fresh assay buffer, and low, medium, or high
20 concentrations of leukocytes (0.75, 1.5, or 3 x 10⁵ cells/ml, respectively) were added per well. Following a 30-minute incubation on a rocking platform at room temperature to allow binding, the slides were inverted and immersed in buffer containing 2% (v/v) glutaraldehyde, such that non-adherent cells were lost and adherent cells were fixed to the monolayer.
25 The adherent mononuclear cells were enumerated using video-light microscopy.

- Monocytes from hypercholesterolemic animals (Chol) exhibited greater adherence, consistent with observation by others, that monocytes from hypercholesterolemic cats or humans exhibit greater adherence to
30 cultured endothelial cells. (deGrujter, et al. (1991) Metabol. Clin. Exp. 40, 1119-1121; Fan, et al. (1991) Virchows Arch. B Cell Pathol. 61, 19-27).

In comparison to monocytes derived from vehicle-treated hypercholesterolemic animals (Chol), those from arginine-treated hypercholesterolemic animals (Arg) were much less adherent. This data shows that the arginine treatment inhibits adhesion of monocytes to the endothelium, which is the first observable event in atherogenesis.

Example 4. Dietary L-Arginine Inhibits the Enhanced Endothelial-Monocyte Interaction In Hypercholesterolemia

The earliest observable abnormality of the vessel wall in hypercholesterolemic animals is enhanced monocyte adherence to the endothelium, which occurs within one week of a high cholesterol diet. This event is thought to be mediated by the surface expression of endothelial adhesion molecules and chemotactic proteins induced by hypercholesterolemia.

Another endothelial alteration that occurs in parallel is a reduced activity of nitric oxide (i.e., NO), derived from metabolism of L-arginine. As shown above chronic dietary supplementation with L-arginine restores NO-dependent vasodilatation in hypercholesterolemic rabbits, and that this improvement in NO activity is associated with a striking anti-atherogenic effect. In the following study was tested the hypothesis that the anti-atherogenic effect of dietary arginine was mediated by endothelial derived NO which inhibits monocyte-endothelial cell interaction.

Methods

Animals. Male New Zealand White rabbits were pair fed, receiving one of the following dietary interventions for 2 weeks: normal rabbit chow (Cont, n = 7); rabbit chow enriched with 1% cholesterol (Chol, n = 7); or 1% cholesterol chow supplemented with 2.25% L-arginine HCl in the drinking water (Arg, n = 7) *ad libitum* throughout the course of the study. In a second series of studies designed to further explore the role of endogenous NO on monocyte-endothelial cell interaction, another group of animals were pair fed, receiving a normal rabbit diet supplemented with either vehicle control (N = 5) or the NO synthase antagonist, nitro-L-arginine (L-NA, 10 mg/100 ml; n = 5),

administered in the drinking water *ad libitum* throughout the course of the study (for an average daily dose of 13.5 mg/kg/day). In a third series of experiments animals received a normal diet and either vehicle (n = 4), L-NA (13.5 mg/kg/day; n = 4), or L-NA and hydralazine (n = 4) added to the drinking water for two weeks. At this dose, hydralazine (5 mg/kg/day) reversed the increase in blood pressure induced by L-NA. One day before sacrifice (after 2 weeks of dietary intervention), animals were lightly sedated and the central ear artery was cannulated for collection of blood samples.

10 *Mononuclear cell culture and isolation.* Murine monocytoid cells, WEHI 78/24 cells were grown in Dulbecco's Modified Eagle's Medium supplemented 10% fetal calf serum (vol/vol) and were kept in an atmosphere of 5% CO₂/95% air. Prior to binding studies, mononuclear cells were fluorescently labeled with TRITC (3 µg/ml). To confirm the results using WEHI cells, in some studies binding studies were performed in parallel using rabbit mononuclear cells. Mononuclear cells were isolated from fresh whole blood of Control rabbits before sacrifice.

20 *Preparation of aortic endothelium and binding assay.* After 2 weeks of the dietary intervention, the thoracic aortae were removed and placed in cold, oxygenated saline. A 15 mm segment of thoracic aorta was excised from a point immediately distal to the left subclavian artery to the mid-thoracic aorta. The segments were then carefully opened longitudinally and placed into culture dishes containing HBSS medium. Aortic strips were fixed to the culture dish using 25 gauge needles so as to expose the endothelial surface to the medium. Culture dishes were then placed on a rocking platform at room temperature.

30 After 10 minutes the HBSS medium was replaced by binding medium containing WEHI cells. The aortic strips were incubated with the mononuclear cells for 30 minutes. The medium was then replaced by fresh binding medium without cells to remove non-adherent cells. The aortic segments were then removed and placed on a glass slide, and adherent cells

counted under epifluorescent microscopy from at least 30 sites on each segment.

Results

5 *Monocyte adhesion to rabbit aortic endothelium.* Exposure of WEHI 78/24 cells to normal rabbit aortic endothelium results in a minimal cell binding in this *ex vivo* adhesion assay. However, when WEHI cells were incubated with aortic endothelium from hypercholesterolemic animals (Chol; n = 7), cell binding was enhanced 3-fold in comparison to Cont (n = 7). The
10 increased cell binding manifested by aortic endothelium of hypercholesterolemic animals was significantly attenuated by L-arginine supplementation (n = 7). (Fig. 3) Similar results were achieved when adhesion assays were performed in parallel with mononuclear cells that were freshly isolated from Cont animals (n = 2) in each of the three

15

Effect of chronic NO synthase inhibition on endothelial adhesiveness.
To further investigate the role of endothelium-derived NO in modulating endothelial-monocyte interaction, an additional series of binding studies were performed using thoracic aorta from animals that received regular chow
20 supplemented with vehicle (n = 5) or the NO synthase inhibitor, L-NA (n = 5). The adhesion of WEHI cells was markedly increased when incubated with aortic endothelium from L-NA animals compared to control endothelium. This effect could not be attributed to hypertension caused by L-NA since concomittant administration of hydralazine normalized blood pressure but did
25 not reverse the augmentation of cell binding induced by L-NA.

 In a separate series of studies it was confirmed that chronic administration of L-NA (the inhibitor of NO synthase) significantly inhibited generation and release of NO from the vessel wall (as measured by chemiluminescence), compared to vessels from animals treated with vehicle
30 or arginine.

 The salient findings of this investigation are: 1) monocyte binding to the endothelium *ex vivo* is increased in vessels from hypercholesterolemic animals; 2) this increase in monocyte binding is attenuated in

hypercholesterolemic animals treated chronically with the NO precursor L-arginine; 3) monocyte binding to the endothelium is increased in vessels from normocholesterolemic animals treated with the NO synthase antagonist L-nitro-arginine; and 4) this effect of NO synthase antagonism was not
5 reversed by administration of hydralazine in doses sufficient to normalize blood pressure. These findings are consistent with the hypothesis that NO inhibits monocyte-endothelial cell interaction.

To conclude, an *ex vivo* model of monocyte binding has been used to study the increase in endothelial adhesiveness induced by
10 hypercholesterolemia. Attenuation of endothelial adhesiveness is attenuated by oral administration of the NO precursor L-arginine is shown. Conversely, inhibition of NO synthase activity by oral administration of nitro-arginine strikingly increases endothelial affinity for monocytes *ex vitro*. The data are consistent with NO being an endogenous anti-atherogenic molecule.

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Example 5. Effect of NO synthase expression on proliferation of vascular smooth muscle cells: Cultured rat aortic vascular smooth muscle cells under confluent quiescent conditions were studied. An efficient viral coat protein-mediated DNA transfer method was employed to transfect the
20 cells with the NO synthase gene driven by the β -actin promoter and CMV enhancer. This resulted in increased NO synthase activity (as measured by the arginine-to-citrulline conversion assay) in comparison to control vector transfected cells. Transfection of the NO synthase gene completely abolished serum-stimulated DNA synthesis compared to control vector
25 transfection. These results indicated that increased expression of NO synthase (associated with increased production of NO) inhibits excessive proliferation of vascular smooth muscle cells. This inhibition can be correlated with treatment of atherosclerosis and restenosis.

30 Example 6. Gene Therapy Using NO Synthase cDNA Prevents Restenosis

The study in Example 5 indicated that NO inhibits proliferation of vascular smooth muscle cells. In atherogenesis and restenosis, excessive

proliferation of vascular smooth muscle cells contributes to lesion formation. Injury to the endothelium in atherosclerosis and after catheter interventions apparently reduces or removes the salutary influence of NO. The following study shows that delivery of the gene for NO synthase to the vessel wall
5 inhibits lesion formation.

A plasmid construct encoding the cDNA of endothelial-type NO synthase (EC-NOS) was synthesized. A full length cDNA encoding for EC-NOS was inserted into the EcoRI site of the pUCcaggs expression vector. Balloon angioplasties of the carotid artery in Sprague-Dawley rats were
10 performed and HVJ-liposomes with plasmids encoding EC-NOS cDNA infused, or plasmids lacking EC-NOS cDNA (control vector) infused. After 4 days to 2 weeks, the rats were sacrificed and the carotid arteries harvested for: 1) histomorphometry; 2) measurement of DNA synthesis; and 3) *ex vivo* determination of NO synthesis and release by bioassay and by
15 chemiluminescence.

Results

Morphometric measurements 2 weeks after injury revealed a significant (68%) reduction of intimal lesion thickness in EC-NOS treated (Inj + NOS) in comparison to control vector treated (Inj + CV) or untreated (Ctr
20 Inj) injured vessels. (Fig. 4) Measurements of DNA synthesis were performed 4 days after injury using bromodeoxyuridine. EC-NOS transfection significantly limited bromodeoxyuridine incorporation (by 25%) in comparison to control vector treated or untreated injured vessels. Vessel segments were studied *ex vivo* using organ chamber technique to bioassay for NO release.
25 Calcium ionophore increases intracellular calcium and activates NO synthase to produce NO. Calcium ionophore induced relaxations in injured carotid arteries transfected with control vector that were only 15% of uninjured vessels. Injured arteries that had been transfected with EC-NOS relaxed to a much greater degree, approximately 50% of that observed in uninjured
30 vessels. Direct measurement of NO (by chemiluminescence) released into the medium revealed that NO released by injured tissues (transfected with the control vector) was only 20% of that released by normal uninjured

tissues. By contrast, injured tissues transfected with EC-NOS released more NO (about 75% of normal).

To conclude, balloon angioplasty of the rat carotid artery removes the endothelial source of NO, induces excessive vascular smooth muscle DNA synthesis and proliferation, resulting in an intimal lesion (restenosis).
5 Transfection of the vessel with EC-NOS at the time of balloon injury partially restores NO production by the vessel, and this is associated with reduced DNA synthesis and vascular smooth muscle proliferation, thereby reducing lesion formation. These results are consistent with the conclusion that NO
10 is an endogenous anti-atherogenic molecule.

Example 7. Exclusion of the Effect of Enhanced Nitrogen or Caloric Balance as Causing the Observed Results:

To exclude an effect of L-arginine on nitrogen or caloric balance as the cause of these results, six animals received 1% cholesterol diet
15 supplemented by additional methionine to increase the dietary methionine six-fold. At ten weeks animals were sacrificed for studies of platelet and vascular reactivity, and histomorphometry. Endothelium-dependent relaxation, platelet aggregation and intimal thickness were not different from those of animals fed 1% cholesterol diet alone. These results reveal that
20 another amino acid, methionine (which is not a precursor of NO) does not mimic the effect of the amino acid L-arginine. Therefore it seems likely that the effect of L-arginine is due to its metabolism to nitric oxide, rather than some other effect of amino acid administration (i.e. change in nitrogen or caloric balance).

25 It is evident from the above results, that by enhancing the nitric oxide levels, by means of nitric oxide precursor compounds or other compounds in the nitric oxide pathway, substantial benefits will ensue to patients with vascular degenerative diseases. This treatment will diminish the formation of atherosclerotic plaque and restenosis, by inhibiting adhesion of monocytes
30 and platelets, and by reducing the proliferation of vascular smooth muscle cells.

By virtue of administering to the host, based on a predetermined regimen, or providing in the host a supply of a component in the synthetic

pathway for production of nitric oxide, so as to maintain a mildly elevated level of nitric oxide in the host, particularly at the site to be treated, the incidence of plaque formation can be substantially diminished. This can be achieved in a variety of ways: by oral administration in accordance with a
5 predetermined regimen of various compounds associated with nitric oxide formation, e.g. L-arginine; by administration at the site, in a predetermined regimen of compounds which can produce nitric oxide, either directly or as a result of physiologic action of endogenous compounds, e.g. enzymes; by employing combinations of compounds, which by their action result in the
10 production of nitric oxide; or the like. These individual administrations, can be done independently or in conjunction with a regimen of other compounds associated with the production of nitric oxide.

Alternatively, one may use genetic engineering to introduce a gene associated with a component in the synthetic pathway for production of
15 nitric oxide, e.g. nitric oxide synthase, where the enhanced production of such compounds will have the effect of driving the equilibrium to an enhanced production of nitric oxide. Thus, the subject invention provides a plurality of pathways to enhance the synthesis or action of nitric oxide, or reduce the degradation of nitric oxide, thereby increasing the effect of
20 endogenous nitric oxide to prevent the formation of vascular lesions and to inhibit restenosis.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by
25 reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be
30 made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method of inhibiting the development of atherosclerosis, or restenosis in the vascular system of a human host susceptible to atherosclerosis or restenosis, said method comprising:
 - administering to said host in accordance with a predetermined regimen a prophylactic or therapeutic dose of an agent other than as a natural food source to enhance the level of endogenous NO in the vascular system.
2. A method according to Claim 1, wherein said agent is a precursor to NO.
3. A method according to Claim 2, wherein said agent is L-arginine or physiologically acceptable salt thereof.
4. A method according to Claim 2, wherein said L-arginine or physiologically acceptable salt thereof is present in a health bar at from about 2 - 25 g.
5. A method according to Claim 1, wherein said agent is an enzyme in the biosynthetic pathway to the production of NO.
6. A method according to Claim 3, wherein said enzyme is expressed as a result of the presence of cells comprising a genetic construct introduced into said cells comprising a gene encoding said enzyme.
7. A method of inhibiting plaque formation in the cardiovascular system of a human host, said method comprising:
 - administering to said host a chemical agent comprising a physiologically acceptable amino compound and a physiologically acceptable oxidant capable of reacting *in vivo* to enhance the level of endothelium-derived relaxing factor in the cardiovascular system.

8. A method according to Claim 7, wherein said amino compound is a guanidino compound and said oxidant is a peroxy compound.

9. A method of inhibiting plaque formation in the cardiovascular system of a human host, said method comprising:

transfecting cells with a genetic construct comprising a gene encoding an enzyme in the biosynthetic pathway to NO and expressed in said cells and secreted;

wherein said cells are either endogenous to said host or introduced into said host;

whereby said enzyme is secreted to enhance the level of NO in the cardiovascular system.

10. A method according to Claim 9, wherein said cells are introduced into said host by vascular injection.

11. A method according to Claim 9, wherein said cells are introduced into said host as a vascular graft seeded with said cells.

12. A method according to Claim 9, wherein said gene encodes NO synthase.

13. A method according to Claim 12, wherein said method further comprises administering to said host in accordance with a predetermined regimen a physiologically acceptable chemical precursor to NO.

14. A method according to Claim 13, wherein said agent is L-arginine or physiologically acceptable salt thereof.

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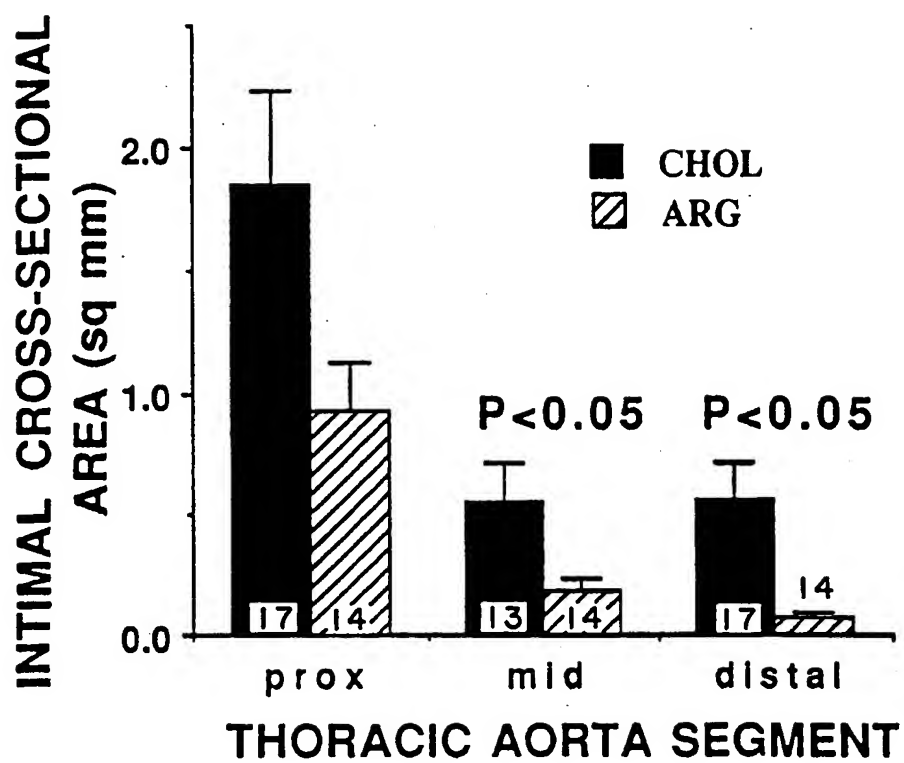
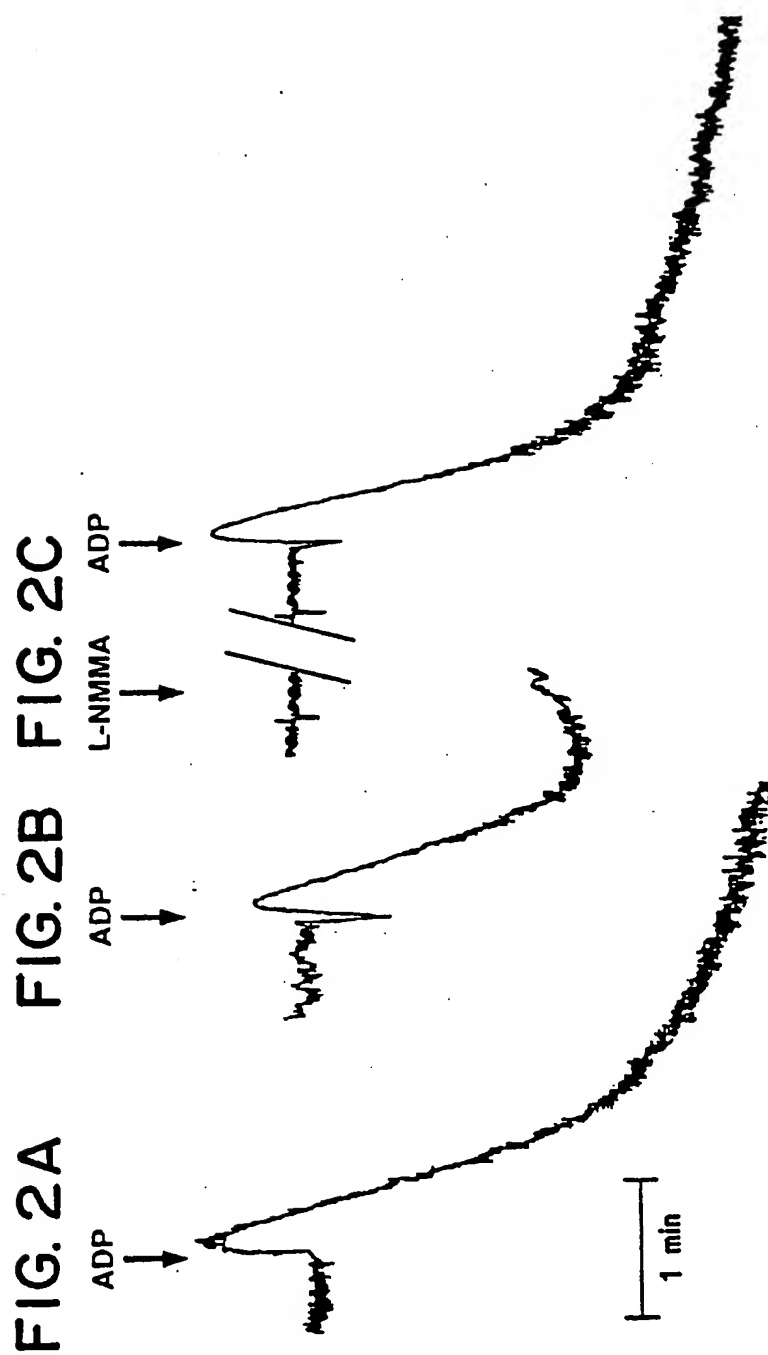


FIG. 1

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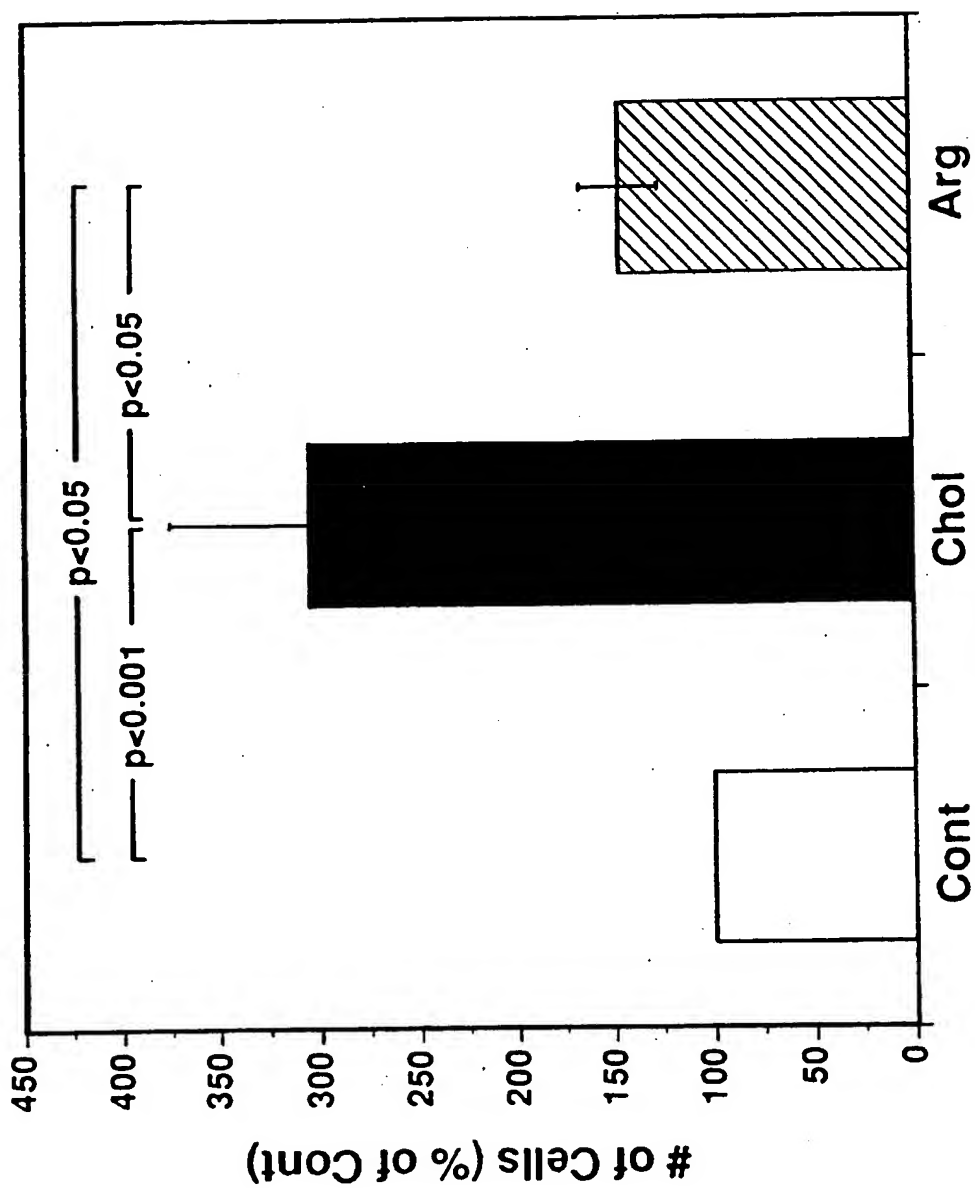


FIG. 3

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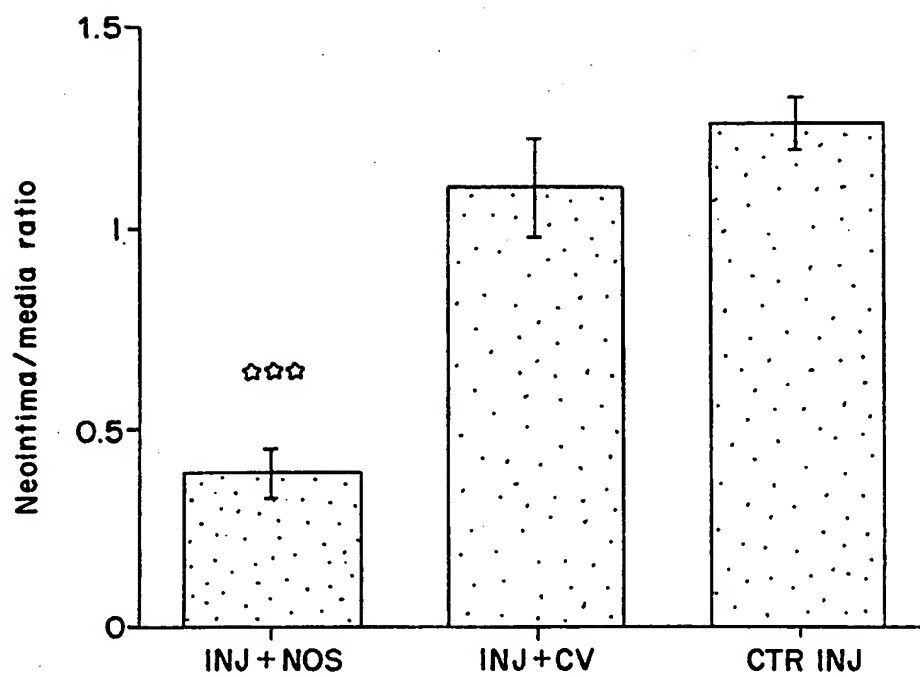


FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06203

| A. CLASSIFICATION OF SUBJECT MATTER IPC(S) : A01N 43/42; A61K 31/47 US CL : 514/310 According to International Patent Classification (IPC) or to both national classification and IPC | | |
|---|--|--|
| B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : A01N 43/42; A61K 31/47; 514/310 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | |
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| A | Nature, Volume 327, issued 21 May 1986 (London), Andrews et al, "Low-density lipoproteins Inhibit endothelium-depend relaxation in rabbit aorta," pages 237-239. See entire document. | 1-14 |
| A | Arteriosclerosis and Thrombosis, Volume 11(2), issued March/April 1991, Bath et al., "Nitric Oxide and Prostacyclin Divergence of Inhibitory Effects on Monocyte Chemotaxis and Adhesion to Endothelium in Vitro," pages 254-260. See entire document. | 1-14 |
| A | The Lancet, Volume 338, issued 21/28 December 1991, Drexel et al., "Correction of endothelial Dysfunction in coronary microcirculation of Hypercholesterolaemic patients," pages 1546-1550. See entire document. | 1-14 |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex. | | |
| * Special categories of cited documents: | T | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
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| Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230 | Authorized officer WERREN LONE jd Telephone No. (703) 308-1235 | |

INTERNATIONAL SEARCH REPORT

International application No.
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| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|---|--|-----------------------|
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| A | Journal of Clinical Investigation, Volume 83, issued May 1989, Garg et al., "Nitric Oxide generating vasodilators and 8-Bromo-Ecyclic Guanosine monophosphate Inhibit Mitogenesis and Proliferation of Cultured Rat Vascular Smooth Muscle Cells, ' pages 1774-1777. | 1-14 |
| A | Circulation Research, Volume 67, No. 6. issued December 1990, Girerd et al., "L-Arginine Augments Endothelium-Dependent Vasodilation in Cholesterol-Fed Rats," pages 1301-1308. See entire document. | 1-14 |
| A | Circulation Research, Volume 54, No. 6. issued June 1984, Heistad et al., "Augmented Response to Vasoconstrictor Stimuli in Hypercholesterolemic and Atherosclerotic Monkeys," pages 711-718. See entire document. | 1-14 |
| A | Proceeding from National Academy of Science USA volume 88, issued June 1991, Kubes et al., "Nitric Oxide an endogenous modulator of leukocyte adhesion", pages 4651-4655. See entire document. | 1-14 |
| A | Nature, Volume 344, issued 08 March 1990, (London) Kugiyama et al., "Impairment of endothelium dependent arterial relaxation by lysolecithin in modified low-density lipoproteins," pages 160-162. See entire document. | 1-14 |
| A | Journal Clinical Investigation, Volume 86, issued December 1990, Minor Jr. et al., "Diet-induced Atherosclerosis increases the RElease of Nitrogen Oxides from Rabbit Aorta," pages 2109-2116. See entire document. | 1-14 |
| A | Circulation Research, Volume 65, No. 6, issued December 1989, Pohl et al., "EDRF increases 6MP in Platels During Passage Through the Coronary vascular Bed," pages 1798-1803. See entire document. | 1-14 |
| A | British Journal Pharmacology, Volume 92, issued May 1987, Radomski et al., "Comparative pharmacology of endothelium-derived relaxing factor Nitric acid and prostacylin in Platelets," pages 181-187. See entire document. | 1-14 |
| A | The New England Journal of Medicine, Volume 314, no. 8, issued 20 February 1986, Ross, "The Pathogenesis of Atherosclerosis," pages 488-500. See entire document. | 1-14 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06203

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-----------------------|
| A | Journal Clinical Investigation, Volume 87, issued April 1991, Rossitch et al., "L-Arginine Normalizes Endothelial Function Cerebral Vessels from Hypercholesterolemic Rabbits", pages 1293-1299. | 1-14 |
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| A | Circulation Research, Volume 66, No. 1, issued January 1990, Tomita et al., "Rapid and Reversible Inhibition by Low Density Lipoprotein of the Endothelium-Dependent Relaxation to Hemostatic Substances in Porcine coronary Arteries," pages 18-27. See entire document. | 1-14 |
| A | Circulation, Volume 81, No. 5, issued May 1990, Weidinger et al., "Persistent Dysfunction of Regenerated Endothelium after Balloon Angioplasty of Rabbit Iliac Artery," pages 1667-1679. See entire document. | 1-14 |
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